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Differential binding of prohibitin-2 to estrogen receptor α and to drug-resistant ERα mutants

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ABSTRACT

Endocrine resistance is one of the most challenging problems in estrogen receptor alpha (ERα)-positive breast cancer. The transcriptional activity of ERα is controlled by several coregulators, including prohibitin-2 (PHB2). Because of its ability to repress the transcriptional activity of activated ERα, PHB2 is a promising antiproliferative agent. In this study, were analyzed the interaction of PHB2 with ERα and three mutants (Y537S, D538G, and E380Q) that are frequently associated with a lack of sensitivity to hormonal treatments, to help advance novel drug discovery. PHB2 bound to ERα wild-type (WT), Y537S, and D538G, but did not bind to E380Q. The binding thermodynamics of Y537S and D538G to PHB2 were favorably altered entropically compared with those of WT to PHB2. Our results show that PHB2 binds to the ligand binding domain of ERα with a conformational change in the helix 12 of ERα.

1. Introduction

Breast cancer is one of the most common diseases in the female population. Nearly 70% of breast tumors express estrogen receptor alpha (ERα) [1]. The C-terminal domain of ERα, the Ligand Binding Domain (LBD), has hormone-dependent transcriptional activity. When an estradiol (E2) molecule binds to the pocket of the LBD, ERα activates transcription, thereby stimulating cell growth [2]. ERα-positive breast cancer is primarily treated with anti-estrogen drugs such as tamoxifen and fulvestrant. However, approximately 30% of this type of breast cancer is unresponsive to such treatment and about 40% of these cancers acquire drug resistance [3]. Moreover, treatment with aromatase inhibitors, which inhibit the biosynthesis of estrogens, can also generate drug-resistant tumors with estrogen hypersensitivity [4].

Several mechanisms of endocrine resistance in breast cancer have been proposed [5]. A point mutation in ERα causes a conformational change that resembles the E2-occupied structure in the LBD, and the mutant acquires almost E2-independent constitutive activity [6]. This mutation further stabilizes the conformation of the E2-occupied ERα and reduces the ligand dissociation rate. Consequently, the ERα mutant activates transcription with E2-hypersensitivity [7]. In fact, although ERα wild-type (WT) is activated to enhance transcription only when stimulated with E2, mutants such as Y537S, D538G, and E380Q show relatively high transcriptional activity even in the absence of E2 stimulation *in vitro* or *in vivo* [5,8,9]. Furthermore, these mutants show low sensitivity to tamoxifen and fulvestrant [8–12]. Accordingly, definitive treatments for these drug- or hormone-resistant breast cancers remains to be established.

The transcriptional activity of ERα is controlled not only by estrogens but also by various estrogen receptor coregulators [13,14]. Repressor of Estrogen receptor Activity (REA), also known as Prohibitin-2 (PHB2), is one such coregulator. PHB2 is thought to translocate from mitochondria to the nucleus when stimulated by E2 and thus regulates the transcriptional activity of ERα [15,16]. As the name suggests, PHB2 is a homolog of PHB1, which was identified in a screen for potential antiproliferative factors [17]. Subsequent studies revealed that PHB2 actually regulates various cellular processes not only in the mitochondria and nucleus, but also in the endoplasmic reticulum, at the plasma membrane, and in the phagosome [18,19]. Accordingly, PHB2 is thought to be a promising therapeutic target for the treatment of various fatal diseases, and breast cancer is one of the most encouraging targets because of the

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extensive studies that have yielded considerable information about ERα. Yet, it is far from clear how PHB2 and ERα interact with each other at the molecular level.

In this study, we characterized the physicochemical properties of recombinant PHB2 protein by using circular dichroism (CD) and differential scanning calorimetry (DSC). The mechanism of PHB2 binding to WT ERα and its mutants was evaluated by using isothermal titration calorimetry (ITC) to study the thermodynamics. We found that PHB2 bound to the Y537S and D538G mutants of ERα and to ERα WT with quite different thermodynamic parameters. Our results suggest that PHB2 binds to both ERα WT and ERα mutants that harbor E2-independent transcriptional activity to repress enhanced transcription.

2. Materials and methods

2.1. Expression and purification of proteins

The gene encoding PHB2 (77–244) was amplified, and the PCR products were inserted into a modified pET vector with a His_6 tag and a TEV protease recognition site. *Escherichia coli* strain BL21 (DE3) (New England Biolabs) transformed with the vector was grown at 37 °C in LB medium supplemented with 50 μg mL^{-1} ampicillin. When OD_{600} reached 0.5, protein expression was induced by adding IPTG at a final concentration of 0.5 mM for 7 h at 20 °C. Cells were harvested by centrifugation and suspended in 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 10% glycerol, and 20 mM imidazole. This solution was then sonicated and centrifuged at 4 °C.

PHB2 was purified by using immobilized nickel affinity chromatography with Ni-NTA agarose (Qiagen). The eluted protein, in 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 10% glycerol, and 250 mM imidazole, was mixed with His_6-tagged TEV protease and dialyzed at 4 °C overnight in buffer containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 1 mM DTT, and 1 mM EDTA. The solution was loaded onto a nickel affinity column to remove uncut PHB2, the cut tags, and the added protease. The flow through was subjected to size exclusion chromatography with a Hiload 26/60 Superdex 200 column (GE Healthcare) pre-equilibrated with 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 10% glycerol. The peak fraction was analyzed by SDS-PAGE, followed by staining with CBB R-250 (Wako).

The ERα LBD (297–554) was cloned into the same pET vector as above, and single mutants (Y537S, D538G, and E380Q) were prepared by using the PrimeSTAR Mutagenesis Basal Kit (TAKARA). Expression and purification of ERα LBD WT and the mutants were performed under the same conditions as those used for PHB2, except for the addition of 20 μM E2 to the purification buffer. ERα LBD WT was prepared both with and without E2.

2.2. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were monitored by using a J-820 spectropolarimeter (JASCO). The measurements were carried out at 25 °C in the wavelength range of 195–260 nm with a scan rate of 20 nm min^{-1} by using a quartz cuvette with 1-mm path-length. Proteins (0.5 mg mL^{-1}) were dissolved in 20 mM HEPES–NaOH (pH 8.0), 150 mM NaCl, 10% glycerol, and 0.5 mM TCEP. The final CD spectra were obtained by taking the average of 5 scans and normalized to the mean residue ellipticity.

2.3. Differential scanning calorimetry

Thermal unfolding of protein was monitored by using a VP–DSC instrument (VP–Capillary DSC, GE Healthcare). Measurements were performed at a protein concentration of 50 μM in 20 mM HEPES–NaOH (pH 8.0), 150 mM NaCl, and 10% glycerol with a heating rate of 1 °C min^{-1}. The thermogram was evaluated by using the ORIGIN software.

2.4. Isothermal titration calorimetry

The interaction between PHB2 and ERα LBD or its mutants was measured with an ITC200 microcalorimeter (GE Healthcare) in 20 mM HEPES–NaOH (pH 8.0), 150 mM NaCl, 5% glycerol, and 0.5 mM TCEP, at 25 °C. The cell was filled with 15 μM E2-bound or unbound ERα LBD WT or E2-bound mutants, and the syringe was filled with 150 μM PHB2. The heat released after injection was determined from the raw data by integration of the individual exothermic peaks after subtraction of the baseline. The binding isotherms were fitted with the ORIGIN software.

3. Results

3.1. Physicochemical properties of PHB2

A partial sequence of human PHB2 including the Band 7 domain and Coiled-coil domain, which is believed to bind to ERα LBD [16], was cloned into a modified pET vector in frame with His_6, TrxA, and a TEV site at the N-terminus (Fig. 1A). The recombinant protein was expressed in the T7 system and purified by using immobilized nickel affinity chromatography, His_6-tagged TEV protease, and size exclusion chromatography (SEC). In the SEC, PHB2 was eluted as a single peak at 234 mL, and the calculated molecular weight at the peak was 22 kDa (Fig. 1B). The purity of the protein was confirmed by SDS-PAGE. The SDS-PAGE gel was stained with CBB, and no contaminating protein was detected (Fig. 1C).

To acquire more information about the structure of PHB2, we measured its circular dichroism (CD) spectrum and performed differential scanning calorimetry (DSC). In the CD analysis, there was a negative band at 208 nm (Fig. 1D), suggesting that PHB2 is an α-helical protein. In contrast, the DSC profile showed that during its thermal denaturation PHB2 absorbed little heat (ΔH = 11 ± 3 kcal mol^{-1} $°C^{-1}$, ΔH_V = 57 ± 11 kcal mol^{-1} $°C^{-1}$), although there was a slight peak at 46 °C (Fig. 1E).

3.2. Binding analysis of PHB2 and ERα LBD with and without E2

To assess the activity of our recombinant PHB2, we analyzed the interaction between ERα LBD WT and PHB2 by using ITC at 25 °C in 20 mM HEPES–NaOH (pH 8.0), 150 mM NaCl, 5% glycerol, and 0.5 mM TCEP. The protein solutions of 15 μM ERα LBD WT and 150 μM PHB2 were placed in a cell and a syringe, respectively. Because PHB2 translocates from mitochondria to the nucleus only in response to stimulation by E2 [20], we hypothesized that PHB2 binds to E2-bound ERα LBD (holo-ERα LBD) WT more strongly than to E2-unbound ERα LBD (apo-ERα LBD) WT. As expected, the holo form of ERα LBD bound to PHB2 with a K_D of 1.3 μM, whereas the apo form showed no binding response (Fig. 2A and B). The thermodynamic parameters of the interaction between the holo ERα LBD and PHB2 are shown in Table 1. This interaction reveals an exothermic reaction with a favorable binding enthalpy change and an unfavorable binding entropy change (ΔG = −8.0 kcal mol^{-1}, ΔH = −13.8 kcal mol^{-1}, $-T\Delta S$ = 5.8 kcal mol^{-1}).

3.3. PHB2 binds to the Y537S and D538G mutants of ERα LBD in a thermodynamically different manner

Three mutations in ERα, Y537S, D538G, and E380Q, were selected on the basis of their frequencies in breast cancer and contributions to the interaction between ERα and its coregulators

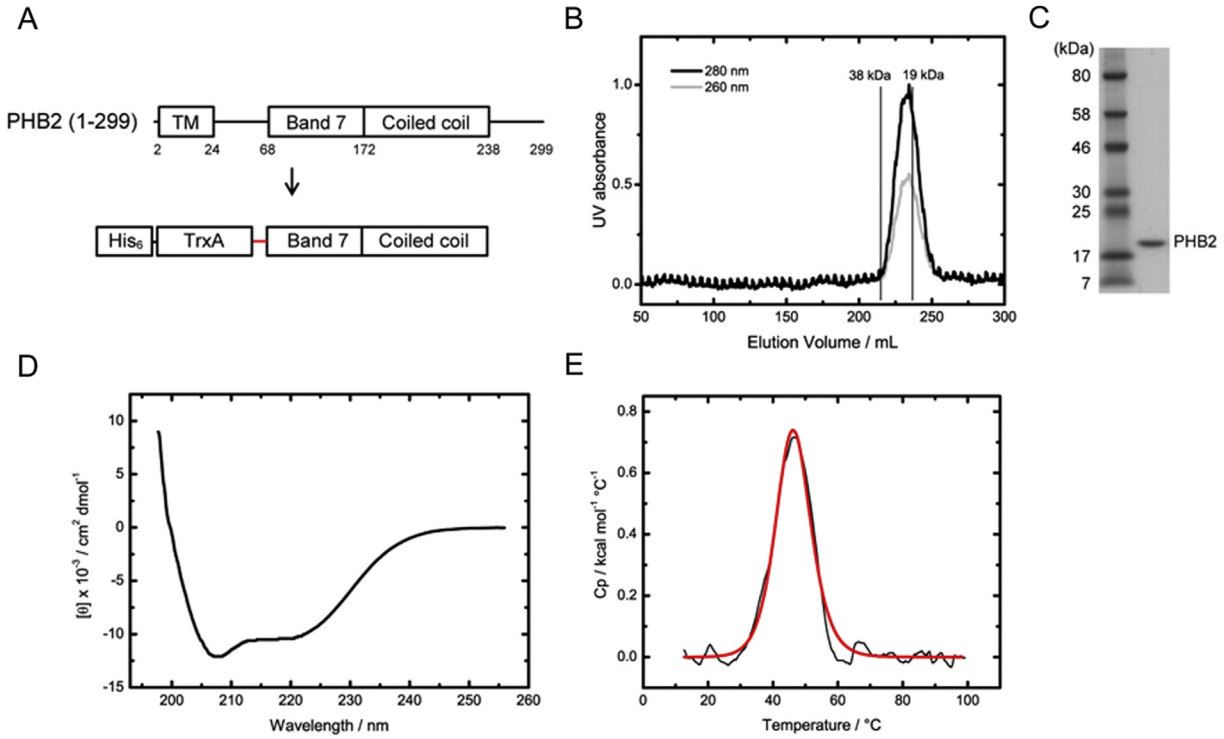

Fig. 1. Physicochemical analyses of recombinant human PHB2. (A) Schematic representation of human PHB2 and the PHB2 domains (Band7 and Coiled coil, 77–244) tagged with His_6 and thioredoxin A (TrxA) at the N-terminus. TrxA and PHB2 are linked by a TEV site. (B) Size exclusion chromatography of PHB2 without a tag. The spectra of UV absorbance at 280 nm and 260 nm are shown in black and gray, respectively. The expected elution volumes of the monomer (19 kDa) and dimer (38 kDa) are indicated. (C) SDS-PAGE of the peak fraction on SEC. Molecular weights of marker proteins are indicated. (D) Circular dichroism spectrum of PHB2. (E) Differential scanning calorimetry thermogram of PHB2. The raw data from which the buffer baseline value was subtracted and the fitting curve calculated by using two-state model are superposed.

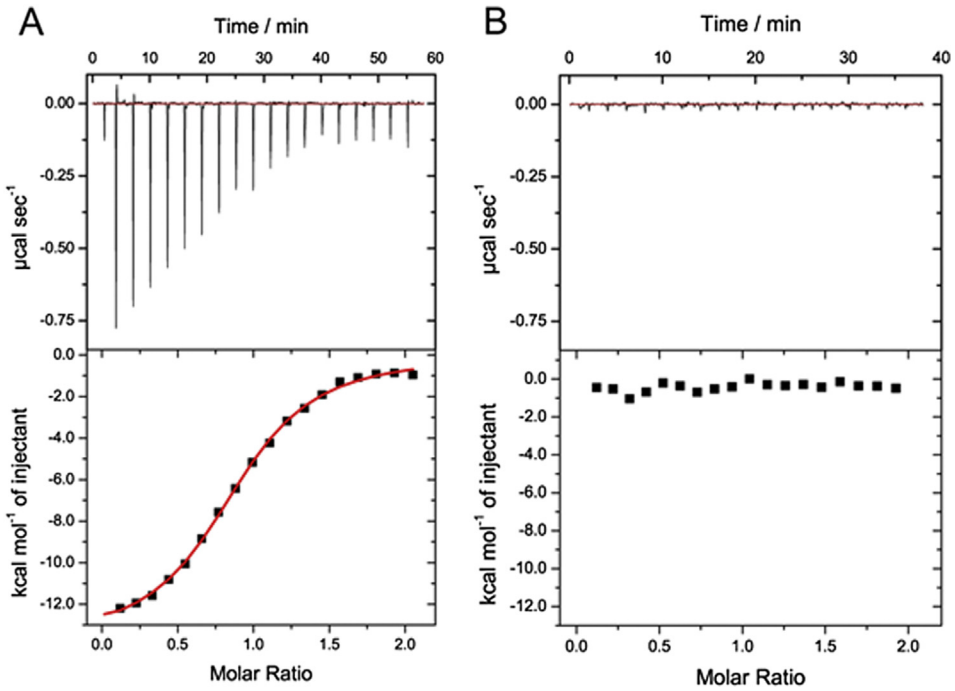

Fig. 2. ITC binding profiles of PHB2 with the apo and holo forms of ERα LBD WT. (A) Titration of E2-bound ERα LBD WT and (B) E2-unbound ERα LBD WT with PHB2. The top and bottom panels correspond to the titration kinetics and binding isotherms, respectively.

Table 1
Affinities and thermodynamic parameters of the PHB2 interaction with ERα LBD WT and ERα LBD mutants.

	N	ΔH (kcal mol^{-1})	$-T\Delta S$ (kcal mol^{-1})	ΔG (kcal mol^{-1})	K_D (μM)
WT	0.90	−13.8	5.8	−8.0	1.3
Y537S	0.96	−4.1	−3.7	−7.8	1.9
D538G	1.12	−6.0	−1.1	−7.1	6.4
E380Q	n. d.	n. d.	n. d.	n. d.	n. d.

N = stoichiometry (PHB2/ERα LBD); n. d. = not detected.

as estimated from the crystal structures of the complexes [5,21]. These three mutants were expressed and purified by the same method used for E2-bound WT.

The interactions between PHB2 and the Y537S and D538G mutants were examined by using ITC under the same conditions as those used for E2-bound WT. While the affinity of Y537S (K_D = 1.9 μM) was similar to that of WT (K_D = 1.3 μM), its thermodynamic profile ($\Delta H = -4.1$ kcal mol^{-1}, $-T\Delta S = -3.7$ kcal mol^{-1}) was quite different from that of WT (Fig. 3C). The point mutation Y537S in ERα modified its binding to PHB2 from entropically unfavorable to entropically favorable ($\Delta\Delta H = 9.7$ kcal mol^{-1}, $-\Delta(T\Delta S) = -9.5$ kcal mol^{-1}). Although the affinity of D538G (K_D = 6.4 μM) was approximately five times that of WT (Fig. 3D), the thermodynamics of D538G ($\Delta H = -6.0$ kcal mol^{-1}, $-T\Delta S = -1.1$ kcal mol^{-1}) showed the same conversion ($\Delta\Delta H = 7.8$ kcal mol^{-1}, $-\Delta(T\Delta S) = -6.9$ kcal mol^{-1}). However, the E380Q mutation did not show any binding heat to PHB2 (Fig. 3E).

3.4. Structural change of ERα LBD mutants binding to PHB2

The CD spectra of WT, Y537S, D538G, and E380Q demonstrated that all of these mutants had the same α-helical secondary structure with a large negative band at around 222 nm, whereas WT had the similar α-helical structure but with a reduced negative band at around 222 nm (Fig. 3A). Furthermore, when we compared the secondary structures of the WT–PHB2 and Y537S–PHB2 complexes in the CD spectra (Fig. 3B), the Y537S–PHB2 complex showed the negative peak at 222 nm derived from the α-helix, but the peak for the WT–PHB2 complex was shifted to 220 nm.

4. Discussion

The SEC profile of PHB2 indicated that the polymeric state of PHB2 was monomeric rather than dimeric (Fig. 1B). Although there are no available three-dimensional structures of PHB2, molecular

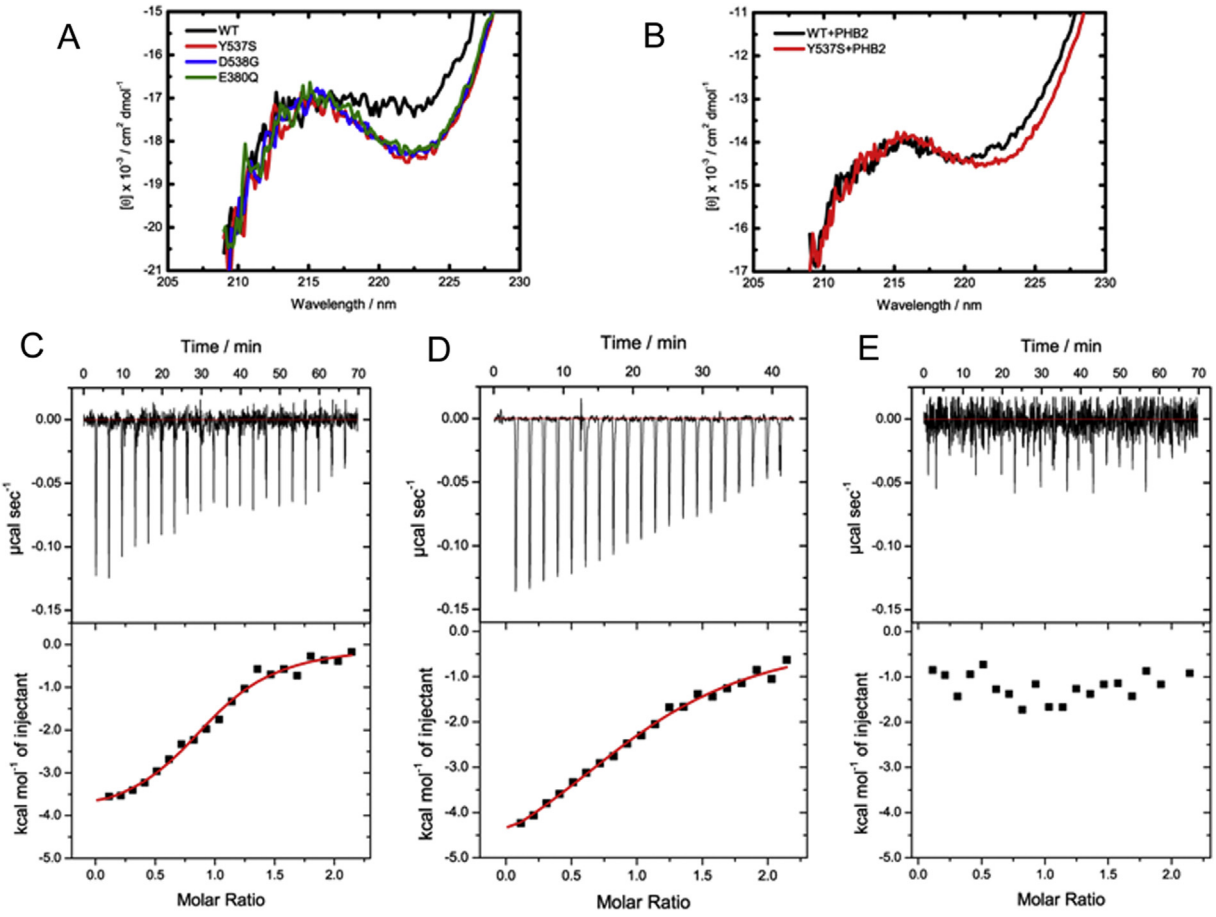

Fig. 3. Interactions of ERα LBD mutants with PHB2. (A) CD spectra of WT, Y537S, D538G, and E380Q mutants are shown in black, red, blue, and green, respectively. (B) CD spectra of the WT–PHB2 and Y537S–PHB2 complexes are shown in black and red, respectively. (C) Titration of E2-bound Y537S, (D) D538G, and (E) E380Q mutants with PHB2. The top and bottom panels correspond to the titration kinetics and binding isotherms, respectively.

modeling has suggested the predicted structure would be rod-like [22]. This structural prediction may explain the slightly early elution of PHB2 in SEC for its molecular weight. In mitochondria, PHB2 forms membrane-bound large ring-like complexes of ~1.2 MDa with prohibitin-1 (PHB1). However, in the cytoplasm or nucleus, the oligomeric state of PHB2 has been unknown. Our results suggest that PHB2 in solution acts as a monomer.

The results obtained from the CD and DSC measurements showed that PHB2 has a clear α-helical structure, whereas its tertiary structure is almost unfolded. The CD spectrum (Fig. 1D) was very similar to that of lysozyme, which contains 39% helix, 11% beta, 34% turns, and 16% coils [23]. In contrast, in DSC, the calorimetric ΔH of lysozyme is 138 kcal mol^{-1} [24], while that of PHB2 was only 11 kcal mol^{-1} (Fig. 1E). Studies on alpha-lactalbumin reveal that the thermal transition in multi-step mechanisms of protein unfolding hardly was detected in DSC, retaining a considerable amount of secondary structure [25]. Proteins harboring these physicochemical properties are sometimes called intrinsically molten globule-like proteins [25,26]. Given that PHB2 is a multifunctional, pleiotropic protein, these characteristics may contribute to its functional polymorphism similar to the versatility in target recognition of intrinsically disordered proteins.

ITC data indicate that the interaction between ERα LBD WT and PHB2 is an exothermic interaction that is specific to E2-bound ERα LBD (Fig. 2A and B), a finding that is in agreement with previous studies [20]. The stoichiometry is nearly 1:1, suggesting that one molecule of ERα LBD binds to one molecule of PHB2. Given that ERα forms a homodimer, PHB2 can be thought to bind to each ERα monomer of the dimer.

In our mutational analyses, ERα WT and its mutants showed different thermodynamic parameters with respect to their binding to PHB2. To explain this phenomenon, we constructed a model for the binding (Fig. 4). First, E380 is considered a hot spot residue for the binding of PHB2, because despite of the CD spectrum of E380Q showing almost the same secondary structure among all of mutants measured (Fig. 3A), E380Q did not bind to PHB2 (Fig. 3E). Next, we focused on the huge loss of entropy in WT. While the helix 12 (H12, shown in green in Fig. 4) of WT adopts the open pocket conformation with high mobility to probe its regulators [7,27], when a coactivator peptide binds, H12 was found to be packed over the body of the LBD [28]. Our study is thus the first to show the *in vitro* binding of a coregulator protein, not a peptide, to ERα LBD and to suggest that a corepressor of PHB2 can also tuck H12 into the pocket, causing it to form a more rigid helix and resulting in the loss of entropy and the relatively high gain in enthalpy with respect to the affinity in the WT. In contrast, in the Y537S and D538G mutants, the closed pocket conformation is dominant [6,7]. Consequently, neither further loss of entropy nor gain of enthalpy would be required for binding to PHB2. This model is supported by comparing the CD spectra of the WT–PHB2 and Y537S–PHB2 complexes (Fig. 3B). The apo WT showed no negative band at around 222 nm, but in WT–PHB2 the negative band shifted to 220 nm and the entire spectrum of the complex was closer in shape. This was not caused simply by PHB2, because the apo PHB2 did not show a negative band at around 222 nm.

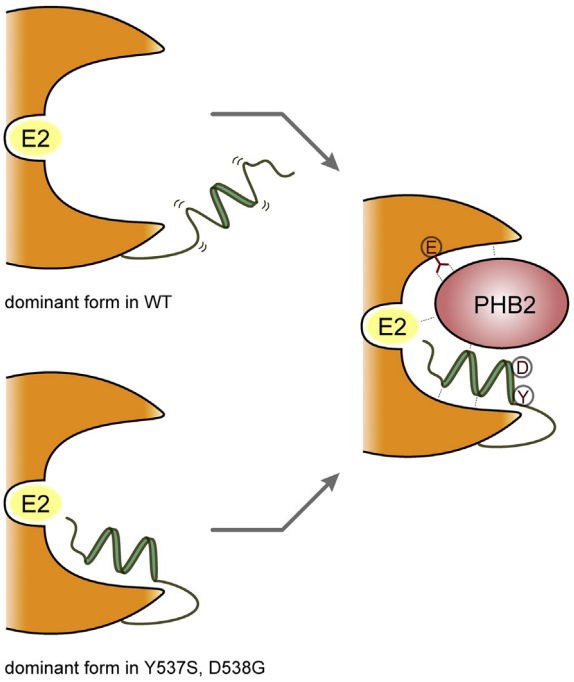

Fig. 4. Schematic model of the binding of PHB2 to ERα LBD. In ERα LBD WT, the flexibility of helix 12 (H12, shown in green) is lost by the binding of PHB2, whereas the mutations at Y537S or D538G fix H12 in the closed form prior to PHB2 binding. E2, E, Y and D are abbreviations for estradiol, glutamic acid 380, tyrosine 537 and aspartic acid 538, respectively.

In the absence of E2, nuclear receptor corepressor (N-CoR) and histone deacetylase (HDAC) are bound to ERα to silence its activity. When stimulated by E2, the activated ERα releases the repressor proteins and acetyltransferases and steroid receptor coactivator (SRC) complexes are recruited to ERα [13]. If this process is also essential for initiating transcription in breast cancer cells, which express mutated ERα, to enhance cancer growth, the above-mentioned coactivators should be able to bind to the ERα mutants. The binding sites of ERα to N-CoR and SRC are in the same pocket and both coregulators form very similar ERα-helix-mediated protein–protein interactions with ERα [28,29]. Although the structure of the ERα-PHB2 complex has not been solved, detailed mutation studies have identified an essential region of the PHB2 sequence that interacts with ERα [16]; this region contains a CoRNR motif-like sequence, which is conserved among nuclear receptor corepressors, and is embedded in the ERα-helix that is involved in the interaction of corepressors with nuclear receptors [29]. Our results demonstrate that PHB2 could bind to some ERα mutants and may suggest that PHB2 fights breast cancer cells that express ERα mutants.

Many previous studies have concentrated on the development of novel endocrine therapies [30–32], yet hormonal treatments still carry the risk of side effects and the potential for the development of resistance due to mutations. Our findings shine a light on the potency of PHB2 as a target for drug to combat breast cancer: for example, a compound that enhances the binding of PHB2 and ERα LBD or inhibits the interaction between PHB2 and breast cancer-specific proteins such as BIG3 [33,34]. Our data and further analyses of the interaction between PHB2 and ERα LBD, such as X-ray crystallography of the complex, will provide further insights to drive the discovery of a novel drug that can overcome resistance derived from mutations and has minimal side effects.

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